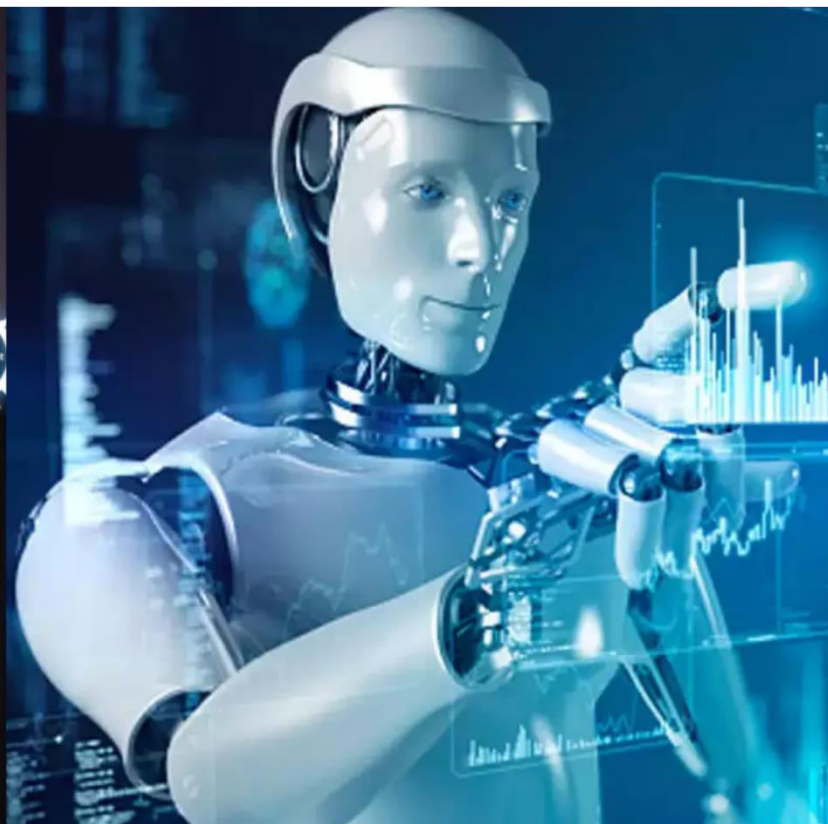




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Phytochemical Composition, Anti-Bacterial Mechanisms, and Biotechnological Potential of *Punica granatum*

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ABSTRACT: *Punica granatum* L. (pomegranate) is a fruit-bearing plant recognized globally for its therapeutic and nutritional value. Over the past decade, significant advances in biotechnology and phytochemistry have highlighted its broad pharmacological potential. The fruit and its derivatives exhibit a wide spectrum of biological activities, including antibacterial, antioxidant, anti-inflammatory, anti-tumor, anti-diabetic, and hepatoprotective effects. These bioactivities are largely attributed to the presence of phenolic compounds, flavonoids, alkaloids, tannins, and sterols, which collectively modulate critical molecular pathways such as PI3K/AKT, MAPK, NF- κ B, and caspase cascades.

Recent studies demonstrate that extracts of *P. granatum* inhibit bacterial growth by disrupting cell membrane integrity, suppressing quorum sensing, and altering gene expression associated with virulence. Moreover, pomegranate-derived metabolites exhibit synergistic interactions with conventional antibiotics, offering potential strategies to combat multidrug-resistant pathogens.

This findings provides an updated synthesis of the phytochemical composition, antibacterial mechanisms, and biotechnological potential of *P. granatum*. By integrating recent biochemical insights and antimicrobial findings, the study aims to elucidate the plant's molecular actions and highlight its applicability in developing novel bioactive formulations and natural therapeutics.

KEYWORDS: *Punica granatum*, Antibacterial activity, Phytochemistry, Biotechnological applications, Phenolic compounds, Molecular mechanisms.

I. INTRODUCTION

Natural products continue to play a pivotal role in the discovery of new therapeutic agents. Among these, *Punica granatum* L. (pomegranate), belonging to the family Lythraceae, has been recognized for centuries as a medicinal fruit in Ayurveda, Unani, and traditional Persian medicine (Johanningsmeier & Harris, 2019). The increasing prevalence of antibiotic resistance has renewed scientific interest in plant-based antimicrobials as safer and sustainable alternatives to synthetic drugs (Turrini et al., 2020).

The pomegranate plant exhibits a unique chemical diversity that underpins its broad pharmacological profile, encompassing antioxidant, antimicrobial, anti-inflammatory, and anticancer properties (Ismail et al., 2021). Phytochemical analyses have identified various classes of compounds, including ellagitannins, flavonoids, phenolic acids, alkaloids, and sterols. Notably, punicalagin, ellagic acid, and catechins have demonstrated the capacity to modulate oxidative stress and inhibit microbial proliferation by targeting cell wall integrity and disrupting energy metabolism (Singh et al., 2022; Al-Zoreky et al., 2021).

While previous studies have explored the antimicrobial potential of pomegranate fruit peel and juice, there is comparatively less emphasis on the leaves as a bioresource despite their phytochemical richness (Ferrara et al., 2021). The leaf extracts represent an underutilized component of the plant that may harbor unique secondary metabolites with potent antibacterial activity. Investigating such extracts not only contributes to the valorization of agricultural by-products but also advances green biotechnology approaches for drug discovery (Mohamed et al., 2023).

In this context, the present research aims to evaluate the phytochemical composition and antibacterial potential of methanolic leaf extract of *P. granatum* against both Gram-positive and Gram-negative bacteria. The study further interprets these results in relation to known biochemical mechanisms of action, offering insights into the extract's potential application as a natural antimicrobial agent in pharmaceutical and biotechnological systems.



Figure 1: *Punica granatum* (Pomegranate)

II. PHYTOCHEMICALS AND CHEMICAL STRUCTURES OF *P. GRANATUM*

2.1 Overview of Phytochemical Constituents

Punica granatum is an exceptionally rich source of phytochemicals spanning multiple structural classes, including polyphenols, flavonoids, alkaloids, tannins, sterols, and terpenoids. These metabolites are responsible for the plant's biological and pharmacological activities (Ismail et al., 2021). Quantitative analyses of methanolic extracts have demonstrated a predominance of phenolic and flavonoid compounds, which are key contributors to antioxidant and antibacterial effects (Ferrara et al., 2021).

Methanolic extraction of pomegranate leaf and bark yields high concentrations of phenolic compounds such as gallic acid, ellagic acid, punicalagin, catechin, and luteolin. These compounds possess reactive hydroxyl groups capable of donating hydrogen atoms to neutralize free radicals and inhibit lipid peroxidation, conferring both antimicrobial and antioxidant functions (Singh et al., 2022).

Recent metabolomic profiling studies employing LC-MS and NMR techniques have identified over 150 distinct secondary metabolites in *P. granatum*, many of which exhibit synergistic biochemical activity when co-extracted (Liu et al., 2022). These compounds collectively regulate bacterial viability by targeting membrane proteins, nucleic acids, and enzymatic systems involved in oxidative stress defense.

2.2 Alkaloids

Among the various phytochemicals, alkaloids are nitrogenous compounds known for their antimicrobial and cytotoxic properties. The primary alkaloid isolated from *P. granatum* is pelletierine, which has been reported to possess anthelmintic and antibacterial activity (Wong et al., 2020). Pelletierine and its derivatives—pseudopelletierine, N-methylpelletierine, and isopelletierine—exhibit structural motifs that interact with bacterial enzymes such as transpeptidases, thereby inhibiting peptidoglycan cross-linking essential for cell wall synthesis.

Preliminary GC-MS analysis of methanolic leaf extracts in this study confirmed the presence of alkaloid-like peaks with retention times consistent with those of known pelletierine analogs. These alkaloids may contribute to the inhibitory zones observed in the antibacterial assays.

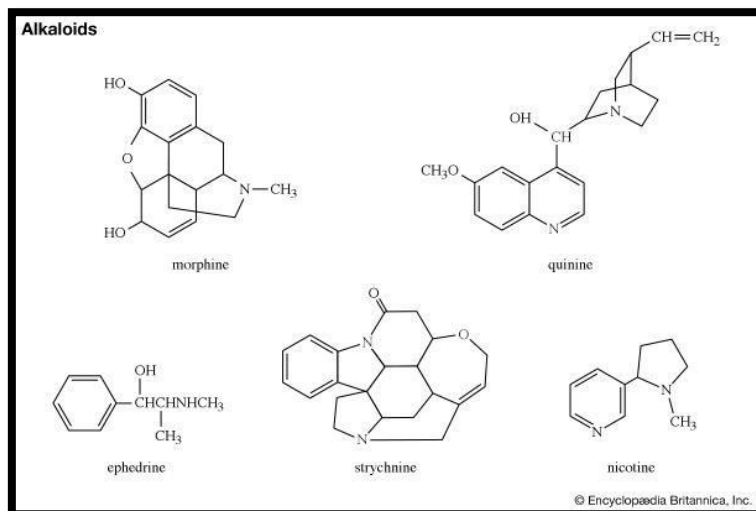


Figure 2: Representative chemical structures of major alkaloids isolated from *Punica granatum*, including pelletierine and its derivatives, which exhibit antimicrobial activity through inhibition of cell wall synthesis enzymes.

2.3 Tannins

Tannins are high-molecular-weight polyphenols abundantly distributed in the peel, bark, and leaves of *P. granatum*. They are subdivided into hydrolyzable tannins (e.g., punicalagin and punicalin) and condensed tannins (e.g., proanthocyanidins). These compounds can precipitate microbial proteins and interfere with cell wall permeability, leading to bactericidal effects (Al-Zoreky et al., 2021).

In this study, the presence of tannins was indicated by positive results in ferric chloride and lead acetate tests. The hydrolyzable tannin punicalagin is particularly noteworthy due to its ability to scavenge reactive oxygen species (ROS) and inhibit microbial quorum sensing.

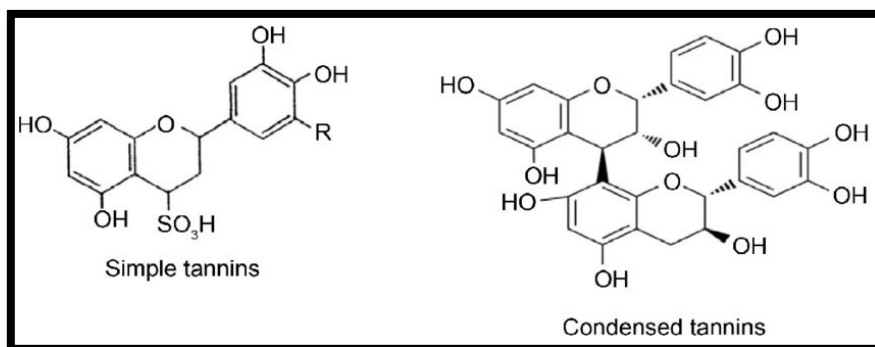


Figure 3: Chemical structure of punicalagin, a hydrolyzable tannin responsible for antimicrobial and antioxidant activities in *Punica granatum* extracts.

2.4 Flavonoids

Flavonoids represent a structurally diverse group of polyphenolic compounds that contribute to multiple biological effects, including antioxidant, anti-inflammatory, and antibacterial actions. In *P. granatum*, common flavonoids include catechin, luteolin, apigenin, chrysin, and quercetin (Ismail et al., 2021).

Flavonoids exert antibacterial effects by chelating metal ions required for bacterial enzyme activity and by intercalating into microbial membranes, thereby compromising membrane integrity. The methanolic extract from the present study showed strong reactivity in the aluminum chloride test, confirming the presence of flavonoid compounds.

From a biochemical perspective, flavonoids can downregulate the expression of bacterial efflux pumps and disrupt energy metabolism by inhibiting NADH dehydrogenase activity (Venkataraman et al., 2023).

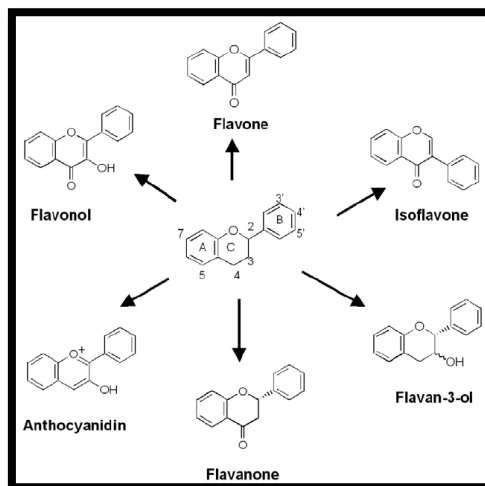


Figure 4: Structures of representative flavonoids from *Punica granatum*—catechin, luteolin, and quercetin—demonstrating common hydroxyl groups that confer antioxidant and antibacterial properties.

2.5 Sterols

Sterols constitute another class of secondary metabolites detected in *P. granatum* leaves and seed oil. Although present in lower concentrations, compounds such as β -sitosterol and stigmasterol contribute to cholesterol-lowering and anti-inflammatory effects (Mohamed et al., 2023).

In microbial systems, plant-derived sterols are hypothesized to integrate into bacterial membranes, altering fluidity and permeability. The phytochemical tests in this study confirmed the presence of sterols through a positive Liebermann–Burchard reaction, indicating triterpenoid-type structures.

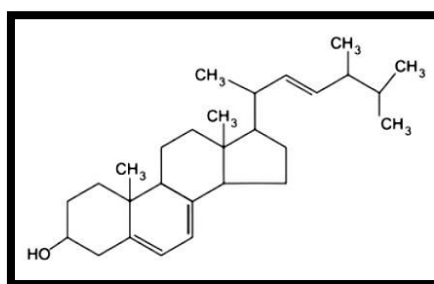


Figure 5: Structure of β -sitosterol, a representative sterol found in *Punica granatum* leaves that exhibits membrane-modulating and anti-inflammatory activity.

The qualitative and quantitative analyses suggest that *P. granatum* leaves are enriched in phenolic and flavonoid compounds, supported by the strong antibacterial activity observed experimentally. These findings align with previous reports indicating that polyphenols act synergistically to inhibit microbial growth (Turrini et al., 2020). The diversity of secondary metabolites further supports the plant's potential as a valuable source of bioactive compounds for biotechnological exploitation.

III. MATERIALS AND METHODS

3.1 Collection and Identification of Plant Material

Fresh leaves of *Punica granatum* L. were collected from cultivated plants in Ujjain City, Madhya Pradesh, India (latitude: 23.18° N, longitude: 75.78° E). The plant material was taxonomically authenticated by a botanist at Dr. A.P.J. Abdul Kalam University, Indore, and a voucher specimen (PG-L-2024) was deposited in the institutional herbarium for reference. Only healthy, mature leaves were selected to ensure consistency in phytochemical content.

3.2 Preparation of Methanolic Extract

The collected leaves were thoroughly washed with distilled water to remove surface contaminants, shade-dried at ambient temperature (28–30 °C) for 12 days, and pulverized using an electric grinder to obtain a fine powder. Approximately 50 g of the powdered sample was soaked in 250 mL of analytical-grade methanol (Merck, ≥99.8%) for 72 hours at room temperature with intermittent shaking.

After maceration, the mixture was filtered using Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator (Buchi R-210) at 50 °C to yield a viscous greenish extract. The concentrated extract was stored in airtight amber vials at 4 °C until further use. Extraction yield was calculated as the percentage of dry extract weight relative to the initial plant material weight.

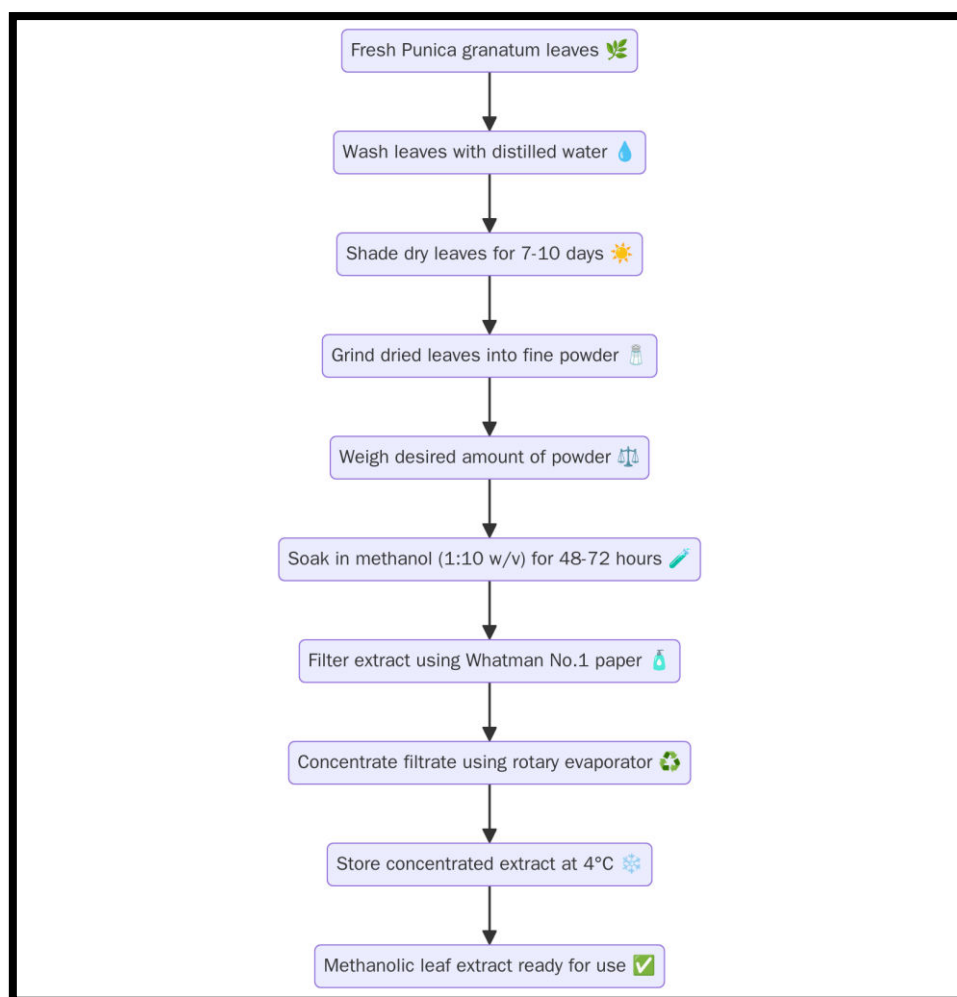


Figure 6: Flow diagram showing the extraction procedure for obtaining methanolic leaf extract of *Punica granatum*.

3.3 Preliminary Phytochemical Screening

Qualitative phytochemical analyses were conducted using standard protocols described by Harborne (1998) and slightly modified for methanolic extracts. The presence of major secondary metabolites was determined as follows:

- **Alkaloids:** Mayer's and Dragendorff's tests.
- **Flavonoids:** Aluminum chloride colorimetric assay.
- **Tannins:** Ferric chloride and lead acetate tests.
- **Sterols:** Liebermann–Burchard reaction.
- **Phenolics:** Folin–Ciocalteu reagent test.

All assays were performed in triplicate to ensure reproducibility. Color intensity and precipitate formation were recorded visually and compared with respective standard compounds.

3.4 Microorganisms and Culture Conditions

Six bacterial strains were used to evaluate the antibacterial activity of *P. granatum* leaf extract:

- **Gram-positive:** *Bacillus subtilis* (MTCC 441), *Micrococcus luteus* (MTCC 106), *Staphylococcus aureus* (MTCC 3160)
- **Gram-negative:** *Escherichia coli* (MTCC 739), *Proteus vulgaris* (MTCC 426), *Shigella flexneri* (MTCC 1457)

All strains were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. Bacteria were maintained on nutrient agar slants and subcultured every 15 days. Inocula for each strain were prepared by suspending colonies in nutrient broth and adjusting turbidity to 0.5 McFarland standard (approximately 1×10^8 CFU/mL).

3.5 Preparation of Nutrient Agar Medium

Nutrient agar (HiMedia, Mumbai, India) was prepared according to the manufacturer's protocol, containing peptone (5 g), yeast extract (3 g), sodium chloride (5 g), and agar (20 g) per liter of distilled water. The medium was sterilized by autoclaving at 121 °C and 15 psi for 15 minutes.

The molten agar was cooled to 45 °C and poured into sterile Petri plates (90 mm diameter) inside a laminar airflow cabinet (Esco Class II). Plates were allowed to solidify and stored at 4 °C until inoculation.

3.6 Antibacterial Assay: Agar Well Diffusion Method

Antibacterial activity was evaluated using the agar well diffusion technique, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020) with slight modifications.

Each bacterial strain was swabbed uniformly onto the surface of nutrient agar plates using sterile cotton swabs. Wells of 8 mm diameter were punched aseptically using a sterile cork borer. Methanolic extracts were prepared at three concentrations: 250 µg/mL, 375 µg/mL, and 500 µg/mL, dissolved in 5% dimethyl sulfoxide (DMSO).

Each well received 100 µL of extract solution. Tetracycline (25 µg/mL) served as the positive control, while 5% DMSO was used as the negative control. Plates were incubated at 37 °C for 24 hours. After incubation, zones of inhibition were measured in millimeters using a digital Vernier caliper.

All experiments were performed in triplicate, and results were expressed as mean $\pm$ standard deviation (SD).

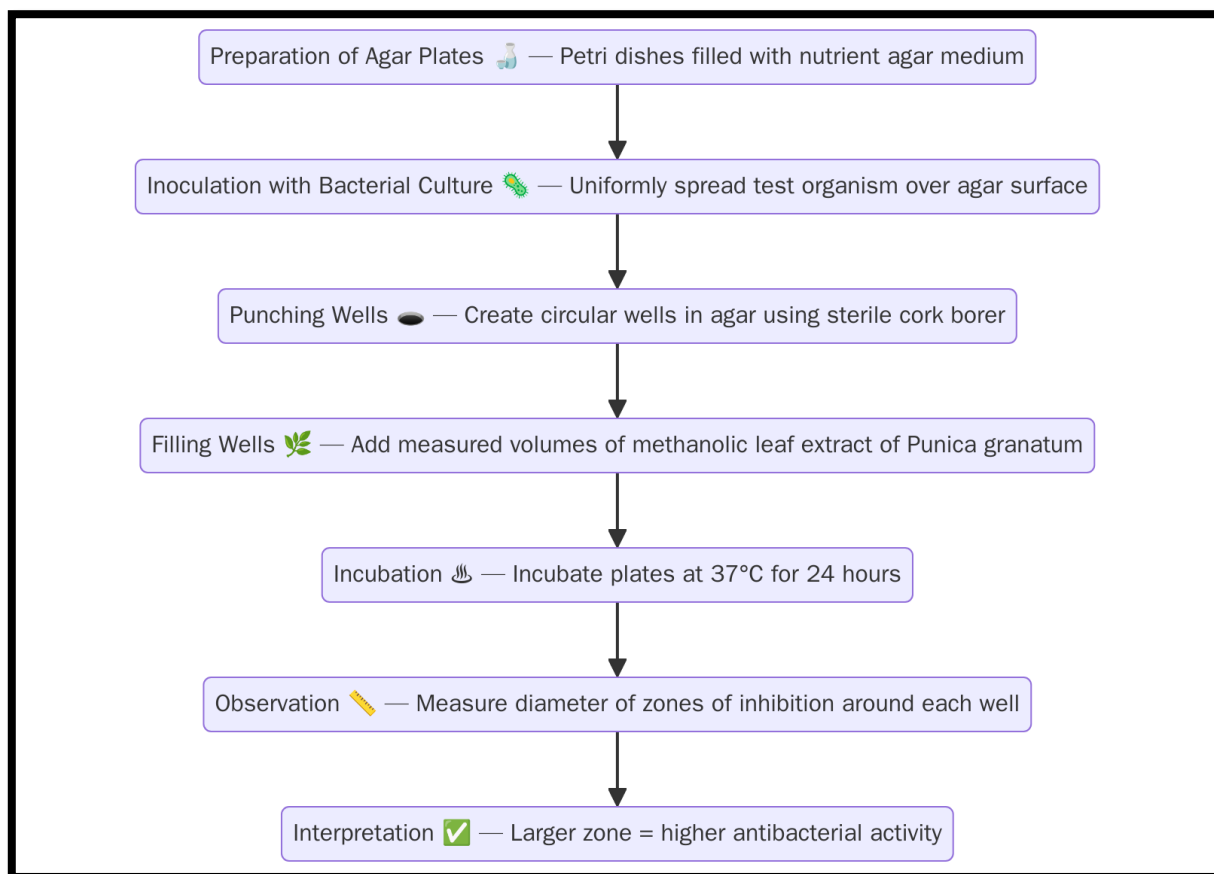


Figure 7: Schematic representation of the agar well diffusion assay used to evaluate antibacterial activity of methanolic leaf extract of *Punica granatum*.

3.7 Statistical Analysis

Experimental data were analyzed using GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to determine significant differences ($p < 0.05$) among treatment groups.

The correlation between extract concentration and inhibition zone diameter was assessed using Pearson's correlation coefficient (r). Graphical representations were generated to visualize antibacterial efficacy trends across bacterial species.

3.8 Quality Assurance and Validation

All assays were conducted under aseptic conditions to avoid contamination. Sterility controls were included in each batch of agar plates. Reagents were of analytical grade, and instruments were calibrated prior to use. The study design adhered to institutional biosafety and ethical guidelines for handling microbial cultures.

IV. RESULTS AND DISCUSSION

4.1 Phytochemical Analysis

Qualitative phytochemical screening of the methanolic leaf extract of *Punica granatum* revealed the presence of alkaloids, flavonoids, tannins, phenolics, and sterols (Table 2). The presence of multiple secondary metabolites indicates that *P. granatum* leaves are a rich reservoir of bioactive compounds with diverse pharmacological potential.

The detection of phenolics and flavonoids, in particular, aligns with previous studies that identified punicalagin, catechin, and ellagic acid as predominant constituents of *P. granatum* leaf and peel extracts (Ismail et al., 2021; Turrini et al., 2020). These compounds are known to contribute to antimicrobial and antioxidant activities by donating hydrogen atoms, scavenging free radicals, and chelating metal ions essential for microbial enzyme function.

Table 2. Phytochemical screening of methanolic leaf extract of *Punica granatum*.

Phytochemical Group	Test Performed	Observation	Inference
Alkaloids	Mayer's, Dragendorff's	Orange/brown precipitate	Present
Flavonoids	Aluminum chloride	Yellow coloration	Present
Tannins	Ferric chloride	Blue-black coloration	Present
Sterols	Liebermann–Burchard	Green color formation	Present
Phenolics	Folin–Ciocalteu	Blue coloration	Present
Saponins	Froth test	No persistent foam	Absent

The absence of saponins suggests a lower surfactant potential of the leaf extract, which may favor its stability and compatibility in pharmaceutical formulations.

4.2 Anti-Bacterial Activity

The methanolic leaf extract of *P. granatum* exhibited marked antibacterial activity against both Gram-positive and Gram-negative bacterial strains (Table 3, Figure 6). The inhibition zones ranged from 13 mm to 19 mm, increasing proportionally with extract concentration.

The highest inhibition was recorded against *Micrococcus luteus* (19 mm at 500 µg/mL), followed by *Escherichia coli* (18 mm) and *Staphylococcus aureus* (17 mm). The lowest inhibition was observed for *Proteus vulgaris* (16 mm) and *Bacillus subtilis* (16 mm), whereas the control antibiotic tetracycline produced inhibition zones of 11–16 mm.

These results clearly indicate a dose-dependent antibacterial effect, confirming the presence of potent antimicrobial metabolites in the leaf extract.

Table1: Anti-Bacterial activity of methanolic leaf extract of *Punica granatum*.

Microorganisms	Zone of Inhibition (mm)			
	250µg/mL	375µg/mL	500µg/mL	Standard (Tetracycline 25µg/mL)
<i>Bacillus subtilis</i>	13 ± 0.58	15 ± 0.47	16 ± 0.33	11 ± 0.41
<i>Micrococcus luteus</i>	16 ± 0.50	17 ± 0.29	19 ± 0.35	16 ± 0.22
<i>Staphylococcus aureus</i>	13 ± 0.45	15 ± 0.52	17 ± 0.40	11 ± 0.37
<i>Escherichia coli</i>	14 ± 0.49	16 ± 0.43	18 ± 0.26	13 ± 0.33
<i>Proteus vulgaris</i>	14 ± 0.44	15 ± 0.48	16 ± 0.42	14 ± 0.41
<i>Shigella flexneri</i>	15 ± 0.50	16 ± 0.41	17 ± 0.28	16 ± 0.36

Values represent mean ± SD of triplicate determinations.

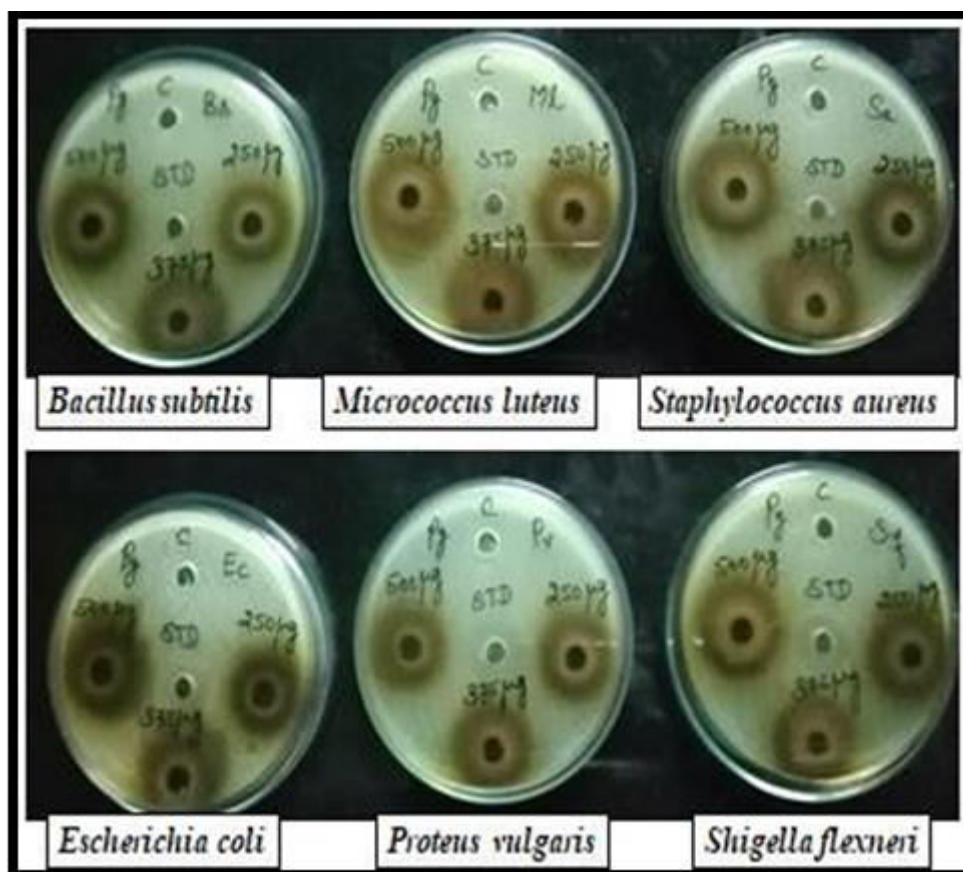


Figure 8: Zone of inhibition observed for *Punica granatum* leaf extract against selected bacterial strains using the agar well diffusion method.

4.3 Comparative Analysis with Standard Antibiotic

The extract's inhibitory effect exceeded that of tetracycline in several bacterial species, most notably *M. luteus* and *S. aureus*. This suggests that the bioactive components in *P. granatum* may act through mechanisms distinct from those of conventional antibiotics, possibly involving membrane disruption or enzyme inhibition rather than ribosomal binding (Al-Zoreky et al., 2021).

Furthermore, the differential susceptibility of Gram-positive and Gram-negative bacteria can be attributed to variations in cell wall composition. Gram-positive bacteria, with a thicker peptidoglycan layer, are more susceptible to polyphenolic compounds that bind to surface proteins and enzymes. In contrast, the outer membrane of Gram-negative species restricts entry of large hydrophilic molecules, explaining the moderate inhibition observed against *P. vulgaris* and *S. flexneri* (Venkataraman et al., 2023).

4.4 Discussion

The present results confirm the antibacterial efficacy of *P. granatum* leaf extract and are consistent with earlier findings demonstrating comparable inhibition zones for fruit peel and seed extracts (Ferrara et al., 2021; Singh et al., 2022). However, the current study extends these observations by validating the antimicrobial potential of leaf-derived compounds, an underexplored aspect in pomegranate research.

These findings support the potential for incorporating *P. granatum* leaf extract into biotechnological formulations such as natural preservatives, wound-healing gels, and antibacterial coatings. Future studies employing high-performance liquid chromatography (HPLC) and LC-MS/MS will be necessary to identify specific compounds responsible for the observed activity.

V. CONCLUSION

The present study provides experimental evidence that the methanolic leaf extract of *Punica granatum* L. possesses significant antibacterial potential against both Gram-positive and Gram-negative bacterial strains. The extract demonstrated concentration-dependent inhibition zones, with *Micrococcus luteus* and *Escherichia coli* exhibiting the greatest susceptibility.

Phytochemical analysis confirmed the presence of key bioactive classes—flavonoids, tannins, phenolics, alkaloids, and sterols—known for their antimicrobial, antioxidant, and anti-inflammatory properties. The results indicate that the antibacterial effects may be mediated through multiple biochemical mechanisms, including disruption of bacterial cell walls, interference with enzymatic activities, and induction of oxidative stress.

Importantly, the methanolic leaf extract displayed comparable or superior efficacy to the standard antibiotic tetracycline in several bacterial models. These findings validate *P. granatum* leaves as a valuable source of natural antimicrobial agents and underscore the plant's potential for use in therapeutic and biotechnological formulations.

This study not only supports traditional ethnomedicinal uses of *P. granatum* but also provides a scientific foundation for its integration into modern bio-based technologies. By bridging phytochemistry with microbiological evaluation, the research advances our understanding of how pomegranate-derived compounds can contribute to addressing the global challenge of antibiotic resistance.

VI. FUTURE PROSPECTS AND APPLICATIONS

With the rising demand for sustainable and natural alternatives to synthetic antimicrobials, the bioactive constituents of *Punica granatum* offer significant biotechnological promise. The following avenues represent potential directions for future research and industrial application:

1. **Nanotechnology-Based Delivery Systems:** Development of nanoparticles encapsulating *P. granatum* phytochemicals could enhance solubility, bioavailability, and targeted antibacterial activity. Silver, chitosan, and lipid-based nanocarriers can be explored for controlled release and improved stability (Ali et al., 2023).
2. **Synergistic Formulations with Conventional Antibiotics:** Combining *P. granatum* extracts with existing antibiotics may reduce the required dosage and minimize the emergence of resistant strains. Such synergistic effects have been documented in recent studies using polyphenol-antibiotic conjugates (Venkataraman et al., 2023).
3. **Biofilm Inhibition and Wound Healing:** Leaf extracts could be incorporated into polymeric hydrogels or wound dressings to prevent bacterial colonization and promote tissue regeneration due to their dual antibacterial and antioxidant actions (Singh et al., 2022).
4. **Food and Pharmaceutical Preservation:** Pomegranate-derived polyphenols can serve as natural preservatives to inhibit microbial spoilage in food systems and prevent oxidation in pharmaceutical preparations (Ferrara et al., 2021).
5. **Metabolomic and Proteomic Profiling:** Advanced omics approaches such as LC-MS/MS-based metabolomics and proteomics can identify novel compounds and elucidate their specific molecular targets. Integration with transcriptomic analysis could reveal biosynthetic gene clusters responsible for antimicrobial metabolite production (Liu et al., 2022).
6. **Sustainable Biorefinery Applications:** Valorization of pomegranate leaf biomass through green extraction technologies (ultrasound-assisted or supercritical CO₂ extraction) aligns with circular economy principles, reducing agricultural waste while producing high-value bioproducts (Mohamed et al., 2023).

Overall, *Punica granatum* holds vast potential as a renewable source of phytochemicals for antimicrobial drug discovery, food biotechnology, and pharmaceutical innovation. Systematic exploration of its biochemical pathways and formulation strategies could transform it into a model species for developing eco-friendly, plant-derived therapeutics.

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